REMARKS

Applicants thank the Examiner for withdrawing the double patenting rejection over U.S. Patent 5,998,205 and for his helpful comments throughout the Office Action.

Specification Amendments

Applicants have amended the specification to correct the spelling of the DF3 promoter in paragraph 141. This amendment is not new matter as the specification contains multiple references to the DF3 promoter (*see, e.g.*, paragraphs 74, 75, 163, 225 and 226), including a second reference to a properly spelled DF3 promoter within amended paragraph 141, itself.

Claim Amendments

Applicants have amended claims 38, 40, 51, 54 and 63.

Applicants have amended claims 40 and 54 to recite a promoter selected from the group consisting of an MUC1/DF3 promoter, an alpha-fetoprotein promoter, an erb-B2 promoter, and a surfactant promoter. Applicants have amended claims 38, 47, 51 and 63 for clarity and form.

Applicants have amended these claims specifically without prejudice and expressly reserve their right to file divisional or continuation applications claiming the cancelled subject matter.

Claims 38-96 are pending.

Rejections

A. Enablement – § 112, first paragraph

Claims 38-96 stand rejected under 35 U.S.C. § 112, first paragraph, as allegedly not enabled for virions comprising a tissue specific replication-conditional adenoviral vector comprising a heterologous tissue specific promoter/enhancer selected from the group consisting of a thymidine kinase promoter, MUC1/DF3 promoter, a p21 promoter, and a cyclin promoter. Without agreeing with the Examiner and only to advance prosecution, applicants have amended claims 40 and 54, the only two claims that specifically recite in words these promoters, to remove the recitation of a thymidine kinase promoter, a p21 promoter, and a cyclin promoter. This amendment overcomes the rejection except as to the MUC1/DF3 promoter.

Applicants request that the Examiner reconsider and withdraw the rejection as to the MUC1/DF3 promoter. First, the Examiner has stated that "the claimed promoters, α-fetoprotein promoter, erb-B2 promoter, and a surfactant promoter are enabled for tissue specific control of adenoviral replication" because the specification states "[a] tissue-specific promoter may be, but is not limited to, AFP, PSA, CEA, DE3, α-fetoprotein, Erb-B2, surfactant, and the tyrosinase promoter." (Office Action, page 4, *citing* page 10, paragraph 141.) Applicants have amended the paragraph pointed to by the Examiner (page 10, paragraph 141) to correct a spelling error to clarify that "DE3" should be "DF3". As demitted above, this correction is supported throughout the application as filed.

Second, the Examiner points to Patton, et al., *Biochimica et Biophysica Acta*, 1241:418, section 7.2 Organs/Tissues (1995) (hereinafter "Patton"), for its alleged teaching that MUC1 is expressed in multiple tissues. The Examiner thus concluded that the "MUC1 enhancer is not actually tissue specific." (Office Action, page 5.) As a preliminary matter, none of the claims recite a MUC1 enhancer. Claims 40 and 54 only recite MUC1/DF3 promoters. Moreover, to the extent that the Examiner's remarks pertain to MUC1/DF3 promoters, applicants disagree that the MUC1/DF3 promoter is not tissue specific. Indeed, other documents that, like Patton, are dated after the application's priority date show that MUC1 in commonly considered tissue specific. *See, e.g.,* Gupta, et al., *Gene Therapy*, 10: 206-212 (Feb. 2003) and Kurihara, et al., *The Journal of Clinical Investigation*, 106(6): 763-771 (Sept. 2000) (both of these documents are enclosed). Accordingly, the weight of the prior art is that MUC1 is tissue specific.

Independent claims 38 and 51 are a genus claims that do not recite specific promoters. These genus claims are also enabled because applicants have shown that a sufficient number of species of promoters are enabled, as acknowledged by the Examiner (see page 3 of the Office Action) and explained above.

Applicants respectfully request reconsideration and withdrawal of this rejection.

B. Obviousness – § 103

1. U.S. Patent 5,698,443 (Henderson, et al.) in view of U.S. Patent 5,631,236 (Woo, et al.)

Claims 38-96 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over U.S. Patent 5,698,443 (Henderson, et al.) in view of U.S. Patent 5,631,236 (Woo, et al.). Applicants traverse.

As a preliminary matter, Henderson is not prior art to this application. The Examiner has stated that this application is entitled to its November 28, 1994 priority. (*See* Office Action page 2.) Accordingly, this application's priority date is before Henderson's June 27, 1995 priority date, and Henderson is not prior art to it.

The primary document not being prior art, any combination with the secondary document, Woo, is not proper. For this reason alone, applicants request that the Examiner reconsider and withdraw the rejection.

Woo does not render any of independent claims 38, 43, 51, 59, 60 or 64 obvious because Woo does not teach or suggest a virion comprising a tissue-specific replication-conditional adenoviral vector comprising a heterologous tissue-specific transcriptional regulatory sequence operably linked to a coding region of a gene (or an adenovirus E1a gene) that is essential for replication of said vector, as recited in independent claims 38 and 51; nor isolated cells comprising such virions, as recited in independent claims 43, 59 and 60; nor a method of producing such virions, as recited in independent claim 64. Rather, Woo's adenovirus vector is a replication incompetent vector that has "the

following elements operatively linked for functional expression: a promoter; a 5' mRNA leader sequence; an initiation site; a nucleic acid cassette, containing the sequence to be expressed; a 3' untranslated region; and a polyadenylation signal." (Col. 2, ll. 62-67; *see also* col. 3, ll. 45-48.) Woo does not teach or suggest adenoviral vectors with heterologous tissue-specific transcriptional regulatory sequence operably linked to a coding region of a gene that is essential for replication, which makes the adenoviral vector replication-conditional.

Accordingly, independent claims 38, 43, 51, 59, 60 and 64, and claims 39-42, 44-50, 52-58, 61-63 and 65-96 (which depend directly or indirectly from claims 38, 43, 51, 59, 60 or 64) are not obvious over Woo. Applicants request reconsideration and withdrawal of this rejection.

2. Henderson in view of Brichard, et al., *Journal of Experimental Medicine*, 178: 489-495 (1993)

Claims 38-96 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over U.S. Patent 5,698,443 (Henderson, et al.) in view of Brichard, et al., *Journal of Experimental Medicine*, 178: 489-495 (1993) ("Brichard"). Applicants traverse.

As explained above, Henderson is not prior art. Further, the Examiner cited Brichard for allegedly disclosing a tyrosinase promoter. None of the pending amended claims recites a tyrosinase promoter. Thus, Brichard is not relevant to the obviousness inquiry for any of the pending claims.

3. Henderson in view of Abe, et al., *PNAS*, 90: 282-286 (1993)

Claims 38-96 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over U.S. Patent 5,698,443 (Henderson, et al.) in view of Abe, et al., *PNAS*, 90: 282-286 (1993) ("Abe"). Applicants traverse.

As explained above, Henderson is not prior art. The Examiner cited Abe for allegedly disclosing a DF3 promoter region. Indeed, Abe does not teach or suggest a virion comprising a tissue-specific replication-conditional adenoviral vector comprising a heterologous tissue-specific transcriptional regulatory sequence operably linked to a coding region of a gene (or an adenovirus E1a gene) that is essential for replication of said vector, as recited in independent claims 38 and 51; nor isolated cells comprising such virions, as recited in independent claims 43, 59 and 60; nor a method of producing such virions, as recited in independent claim 64. Accordingly, applicants request reconsideration and withdrawal of this rejection.

4. Henderson in view of Smith, et al., *Human Gene Therapy*, 5: 29-35 (1994)

Claims 38-96 stand rejected under 35 U.S.C. § 103(a) as allegedly obvious over U.S. Patent 5,698,443 (Henderson, et al.) in view of Smith, et al., *Human Gene Therapy*, 5: 29-35 (1994) ("Smith"). Applicants traverse.

As explained above, Henderson is not prior art. The Examiner cited Smith for allegedly disclosing a surfactant promoter region. Indeed, Smith does not teach or suggest a virion comprising a tissue-specific replication-conditional adenoviral vector comprising a heterologous tissue-specific transcriptional regulatory sequence operably

linked to a coding region of a gene (or an adenovirus E1a gene) that is essential for replication of said vector, as recited in independent claims 38 and 51; nor isolated cells comprising such virions, as recited in independent claims 43, 59 and 60; nor a method of producing such virions, as recited in independent claim 64. Accordingly, Applicants request reconsideration and withdrawal of this rejection.

C. Double Patenting

1. U.S. Application 11/601,071

Claims 38-96 stand rejected for provisional obviousness-type double patenting over claims 19-23, 26-34 and 36-40 of U.S. Application 11/601,071. Filing a Terminal Disclaimer at this time is premature. Neither claims 38-96 of this application nor claims 19-23, 26-34 and 36-40 of U.S. Application 11/601,071 have been allowed. Applicants will file a Terminal Disclaimer, if appropriate and required, when the pending claims are allowed.

2. U.S. Patent 6,551,587

Claims 38-96 stand rejected for provisional obviousness-type double patenting over claims 1-87 of U.S. Patent 6,551,587. Filing a Terminal Disclaimer is premature. Claims 38-96 of this application have not been allowed. Applicants will file a Terminal Disclaimer, if appropriate and required, when the pending claims are allowed.

Related Applications

Applicants direct the Patent Office's attention to prosecution history and any Office Actions that have issued in U.S. Patent Applications 08/348,258, 08/487,992, 08/849,117, 08/974,391, 09/210,936, 10/323,425, 10/323,955, 10/323,984, 11/217,386, 11/601,071, 11/977,533, 11/977,902 and 11/977,903.

CONCLUSION

In view of the foregoing, applicants request that the Examiner consider claims 38-96 and allow them. Should the Examiner believe that any remaining issues can be resolved by telephone conference, the Examiner is invited to telephone the undersigned at any time.

Respectfully submitted,

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RESEARCH ARTICLE

Selective gene expression using a DF3/MUC1 promoter in a human esophageal adenocarcinoma model

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The efficacy of replication-deficient adenoviral vectors in gene therapy is confined to the number of tumor cells the vector infects. To focus and enhance the therapeutic efficacy, we employed a conditionally replication-competent adenoviral vector with a tissue-specific promoter, DF3/MUC1, in a human esophageal adenocarcinoma model. Our results demonstrate that Ad.DF3.E1A.CMV.TNF (Ad.DF3.TNF) specifically replicates in Bic-1 (DF3-producing cells) and mediates an enhanced biologic effect due to increased TNF-α in the same DF3-producing cells. We also show that the increased TNF-α interacts with ionizing radiation to produce greater tumor regression and a greater delay in tumor regrowth in Bic-1 (DF3-producing cells) compared to Seg-1 (DF3 non-producers). Tumor cell targeting using conditionally replication-competent adenoviral vectors with tumor-specific promoters to drive viral replication and deliver TNF-α provides a novel approach to enhancing tumor radiosensitivity.
Gene Therapy (2003) **10**, 206–212. doi:10.1038/sj.gt.3301867

Keywords: *df3; muc1; gene therapy; radiation; esophageal cancer*

Introduction

Adenoviruses have utility for gene therapy because these vectors have a wide spectrum of tissue infectivity, can be produced in high titers, and are easily genetically modified.[1,2] Viral vectors function by replicating to toxic levels within infected cells or by delivering a 'suicide gene' encoding enzymes to locally modify systemically administered non-toxic drugs into their toxic metabolites.[3,4] Additionally, therapeutic genes inserted into the vector may be infected into specific host tissues to produce a biological effect.[5,6] However, most viral vectors do not preferentially infect tumor cells. To prevent viral replication and subsequent toxicity to normal tissues, the E1A gene, which is critical for adenoviral replication, is frequently deleted. A replication-deficient vector is one strategy employed to modify adenoviruses for *in vivo* investigations and potential human trials. However, the antitumor efficacy of a replication-deficient adenovirus is limited by the inability to reproduce and spread its progeny to infect neighboring tumor cells.

To circumvent this limitation, tissue-specific promoters have been employed to exploit specific tumor characteristics, such as the expression of tumor-specific proteins.[7] To enhance the selectivity and efficiency of viral replication, transcription segments from the carcinoembryonic antigen (CEA),[8] prostate-specific antigen (PSA)[9] and alpha-feto protein (AFP)[10] genes are inserted upstream of the E1A gene to drive viral replication in tumors responsible for producing the characteristic proteins and thereby convert replication-deficient vectors to conditionally replication-competent vectors. For example, the CEA promoter, activated by transcription factors responsible for CEA production in colon carcinomas and ligated upstream of the adenoviral gene E1A, mediates viral replication selectively within CEA-producing colon carcinoma cells.

The first studies on the control of mammalian cell gene expression by ionizing radiation (IR) were reported by Hallahan *et al*[11,12] and Sherman *et al*.[13,14] The response of c-jun and Egr-1 to IR has been reported in detail by Datta *et al*.[15–17] The general motif for transcriptional induction by IR was determined to be through modification of transcription factors by a cellular kinase and hence binding of these factors to cognate elements in the promoter/enhancer regions of Egr-1 or c-jun.

DF3/MUC1 (DF3) is a high molecular weight glycoprotein that consists of peptide tandem repeats containing threonine and serine residues, which are potential sites for glycosylation.[18] Late in carcinogenesis, hypoglycosylation of DF3 leads to its aberrant overexpression in a wide variety of epithelial cancers including breast, ovary, and gastrointestinal malignancies.[19] We have previously demonstrated that an adenoviral vector can be modified to be conditionally replication competent in a DF3-positive breast carcinoma model.[20] In the present study, we investigated the potential efficacy of the DF3 promoter to selectively enhance viral replication and

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therapeutic gene delivery in DF3-producing esophageal cancer cells. Tumor necrosis factor alpha (TNF-α) was selected as a potential therapeutic gene because of its antitumor and radiosensitizing properties based on investigations of gene therapy with vectors encoding TNF-α combined with IR.[21,22] We also studied the interactive cytotoxic effects of TNF-α and IR *in vivo* in DF3-positive Bic-1 and DF3-negative Seg-1, human esophageal adenocarcinoma xenografts.

Results

DF3/MUC1 expression in Seg-1 and Bic-1 cell lines

The expression of DF3/MUC1 in the Seg-1 and Bic-1 cell lines was assessed by flow cytometric analysis with an anti-MUC1 antibody (Figure 1). Human embryonic kidney fibroblast 293 cells (a) demonstrated minimal DF3/MUC1 expression compared to the isotype-identical control and therefore served as a negative control. The MCF-7 breast carcinoma cell line (b) exhibited strong DF3/MUC1 expression and served as a positive control. DF3/MUC1 was moderately expressed in Bic-1 human esophageal adenocarcinoma cells (c), but was undetectable in Seg-1 human esophageal adenocarcinoma cells (d).

TNF-α production suggests selective viral replication in DF3/MUC1-producing cells

Bic-1 and Seg-1 human esophageal adenocarcinoma cells were infected *in vitro* with Ad.DF3. TNF at 0.001 MOI and TNF-α production was quantified to indicate viral replication and evaluate TNF-α expression. hTNF-α ELISA of the conditioned medium from Bic-1 cultures and Seg-1 cultures reveals equivalent TNF-α secretion at days 1 and 2 (Figure 2) demonstrating the constitutive activity of the CMV promoter. However, by day 5 the

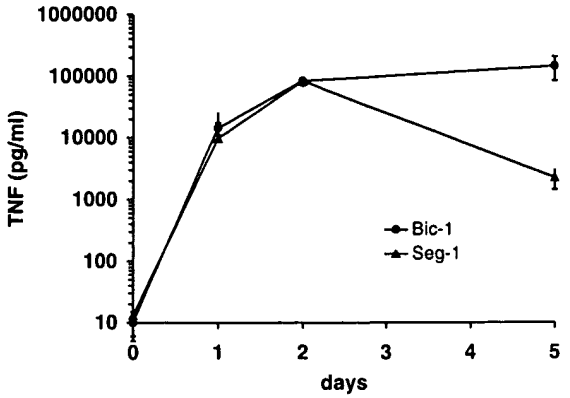

Figure 2 DF3 expression by Bic-1 drives viral replication and selective TNF-α expression in vitro. Conditioned medium (CM) from Seg-1 (▲) and Bic-1 (•) human esophageal adenocarcinoma cells exposed to 0.001 MOI Ad.DF3.TNF was collected on days 0, 1, 2, and 5. hTNF-α ELISA demonstrates significantly greater TNF-α levels in CM from the Bic-1 (DF3/MUC1+) cells compared with CM from the Seg-1 (DF3/MUC1−) cells at day 5 ($P < 0.005$, t-test).

greater than 50-fold increase in TNF-α produced by the Bic-1 (DF3+) cell line compared to the Seg-1 (DF3−) cell line (141 200+60 000 *versus* 2200+800 pg/ml, respectively) suggests enhanced viral replication, increased TNF-α gene expression and, subsequently, greater TNF-α production in the DF3-producing cell line (Bic-1) compared to the non-producers (Seg-1). These findings are in agreement with those published by Kurihara *et al*.[20]

TNF-α production suggests selective viral replication in DF3/MUC1-producing xenografts

Homogenates of Bic-1 and Seg-1 xenografts treated with single intratumoral dose of Ad.DF3. TNF (2×10^8

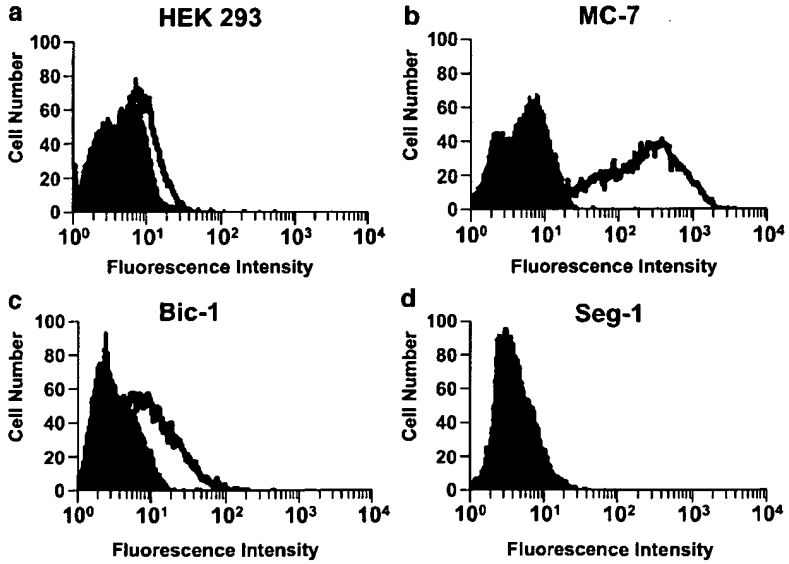

Figure 1 Differential DF3/MUC1 expression in Bic-1 and Seg-1 human esophageal adenocarcinoma cells. DF3/MUC1 expression was determined by flow cytometric analysis following incubation with anti-DF3 monoclonal antibody (open area) or an isotype-identical control antibody (shaded area). The HEK 293 cell line (a) serves as a negative control and MCF-7 breast carcinoma cell line (b) is a positive control. DF3/MUC1 is moderately expressed in Bic-1 (c), but is undetectable in Seg-1 (d) human esophageal adenocarcinoma cells.

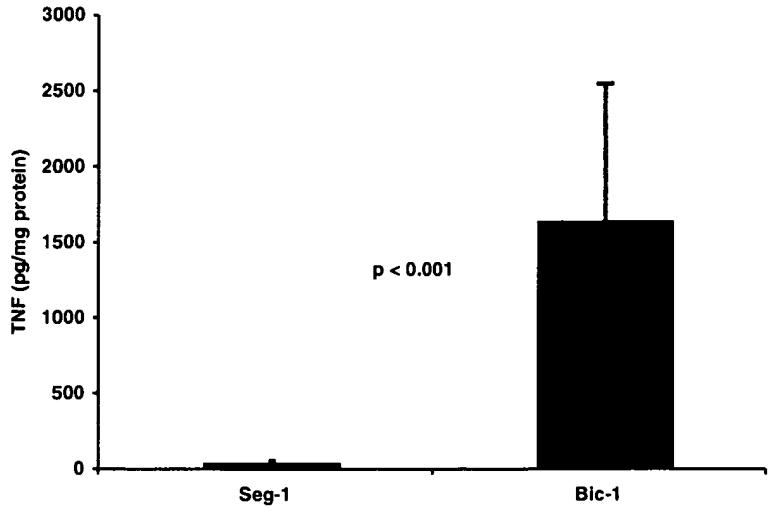

Figure 3 DF3 expression by Bic-1 drives viral replication and selective TNF-α expression in vivo. Seg-1 and Bic-1 xenografts grown in athymic nude mice received a single intratumoral injection of 2 × 10⁸ pu Ad.DF3.TNF in multiple sites. Tumors were harvested on day 7 and tumor homogenates prepared. ELISA data demonstrate significantly higher TNF-α levels in homogenates from Bic-1 (DF3/MUC1+) xenografts compared with Seg-1 (DF3/MUC1−) xenografts (P < 0.001, t-test).

Particle units (pu)) harvested at day 7 demonstrate a 50-fold increase in TNF-α production in the Bic-1 xenografts as compared to the Seg-1 xenografts (range: 804.3–3465.1 *versus* 29.1-37.4 pg/mg of protein, respectively, Figure 3). The broad range in TNF-α levels observed in these studies is related to the number of tumor cells infected by the adenoviral vector following intratumoral injection. It has been documented that vectors do not diffuse within the tumor volume but rather locate along the needle tract. To circumvent this problem, we inject the tumor in multiple sites. The small amount of TNF-α produced in the Seg-1 xenograft homogenates indicates non-selective adenoviral infection and the basal constitutive TNF-α expression under the control of the CMV promoter. Our *in vivo* data support the *in vitro* findings and suggest enhanced viral replication that is indicated by greater TNF-α gene expression in Bic-1 (DF3+) xenografts compared with the Seg-1 (DF3−) xenografts.

Histology suggests enhanced viral replication in DF3/MUC1-producing xenografts

Bic-1 and Seg-1 xenografts treated with either the vector containing a null virus tagged with green fluorescence protein (Ad.DF3. GFP) or the TNF-α gene (Ad.DF3.TNF) were harvested at day 21 and examined with immunofluorescence (Figure 4). Enhanced green fluorescence staining was apparent in Ad.DF3.GFP-treated Bic-1 xenograft sections (b) suggesting continued viral replication as compared to the absence of signal in the Seg-1 xenografts (a). Loss of nuclear architecture following DAPI staining in Ad.DF3.TNF treated Bic-1 sections (d) suggests enhanced viral replication and/or cell death as compared to the normal rounded nuclear appearance in the Seg-1 xenografts (c). These findings further demonstrate the selectivity of the DF3 promoter to direct viral replication and therapeutic gene expression within Bic-1 (DF3+) xenografts compared with Seg-1 (DF3−) xenografts.

Enhanced TNF-α production in Bic-1 xenografts increases antitumor effects of IR

To determine the potential effects of enhanced antitumor selective viral replication and enhanced TNF-α gene expression *in vivo*, we randomized athymic nude mice bearing Seg-1 (DF3−) xenografts to one of six treatment groups. Following administration of treatment, mean fractional tumor volumes were determined (Figure 5a). No significant difference in mean tumor volume was detected between the control groups (buffer, null virus alone, or Ad.DF3.TNF alone). No effect on tumor growth was detected following treatment with IR alone, null virus +IR or Ad.DF3.TNF+IR. These findings suggest a lack of TNF-α gene expression in the Seg-1 (DF3−) xenografts.

By comparison, Bic-1 xenografts (Figure 5b) treated with either the null virus or Ad.DF3.TNF demonstrated a nine-day growth delay compared to buffer-treated controls. However, TNF-α expression in Ad.DF3.TNF-treated xenografts did not alter the regrowth pattern compared to xenografts treated with the null virus. Importantly, combined treatment with Ad.DF3.TNF+IR produced significant tumor regression compared to treatment with Ad.DF3.Null+IR. The combined effect of TNF-α and IR significantly reduced mean tumor volume by 40% after day 22 ($P < 0.05$) and resulted in an increased growth delay of 19 days compared to 7 days in the Ad.DF3.Null+IR group.

Discussion

Analyses of the Surveillance, Epidemiology, and End Results (SEER) database report that the incidence of esophageal adenocarcinoma is rapidly increasing and greatest in caucasian males.[23–25] Risk factors for esophageal adenocarcinoma include gastroesophageal reflux disease, obesity, and Barrett's esophagus. Despite the use of multi-modality therapy, median overall survival remains dismal at 12–15 months. However, a minority

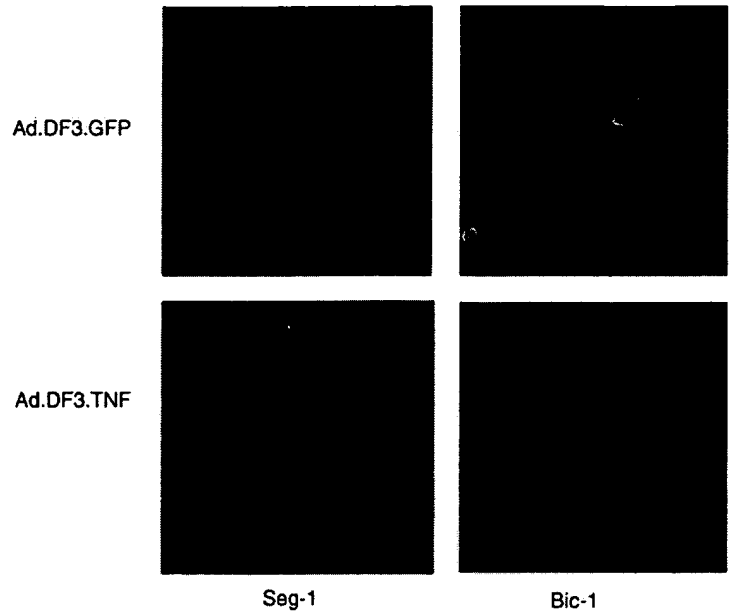

Figure 4 Histological evidence for enhanced viral replication and selective TNF-α expression in Bic-1 xenografts. Seg-1 and Bic-1 xenografts received a single intratumoral injection of either 2x10⁸ pu Ad.DF3.GFP or Ad.DF3.TNF. At day 18, tumors were harvested, fixed, and sectioned. Ad.DF3.GFP-treated Bic-1 (b) tumors demonstrate intense fluorescence staining compared to Ad.DF3.GFP-treated Seg-1 (a) tumors. Ad.DF3.TNF-treated tumors stained for apoptosis with DAPI demonstrated a loss of nuclear architecture in the Bic-1 xenografts (d) compared to the normal rounded nuclear appearance in the Seg-1 tumors (c).

of patients with esophageal adenocarcinoma have a complete pathologic response to preoperative induction chemo-radiotherapy. Subset analyses demonstrate that only true complete responders have a durable disease-free and overall survival benefit.[26–29] The addition of gene therapy to currently employed modalities of chemotherapy, radiotherapy and surgery attempts to enhance the overall antitumor efficacy and increase the number of complete responders.

It has been reported that MUC1 is expressed in as high as 43% of esophageal adenocarcinoma specimens and that a majority of these specimens were poorly differentiated advanced neoplasms.[30] Transcriptional elements from the DF3/MUC1 gene were used to create a tumor-specific viral promoter that recognizes esophageal adenocarcinoma cells that produce DF3/MUC1. Ad.DF3.E1.CMV.TNF is a conditionally replication-competent adenoviral vector that non-selectively infects human and rodent cells, but selectively replicates in DF3/MUC1-producing cells. The expansion of the number of viral particles in a DF3/MUC1-producing cell results in greater TNF-α gene expression that translates into enhanced local TNF-α production. TNF-α has both chemo-sensitizing[31] and radio-sensitizing[32] properties as well as an inherent antitumor effect that is accomplished by coagulative necrosis and capillary destruction.[33] Transcriptional targeting using the DF3 promoter in Ad.DF3.TNF gene therapy drives selective viral replication and enables selective TNF-α gene expression in DF3-producing tumor cells. TNF-α gene therapy focuses delivery of TNF-α to the neoplasm, avoids severe systemic toxicities and therefore may enhance the therapeutic ratio.

We previously demonstrated that TNF-α enhances the antitumor effects of IR in several human tumor cell types.[34] We also reported significant tumor regression following treatment with TNF-α delivered using a replication-deficient adenoviral vector (Ad.Egr.TNF) combined with IR.[35–37] The Ad.Egr.TNF vector used in our studies contained the radiation-responsive DNA sequences of the EGR1 promoter ligated upstream of the cDNA for human TNF-α. The use of the radiation-inducible promoter permitted spatial and temporal control of TNF-α expression within the tumor volume. Tumor cell killing was achieved through a bystander effect and without any increase in normal tissue toxicity when compared with IR treatment alone.[35] With respect to esophageal cancer, the proximity of the esophagus to critical structures, such as the heart and lungs, mandates an even higher specificity of TNF-α expression for the neoplasm while sparing adjacent normal tissues.

Employment of the DF3 tissue-specific promoter in DF3-producing tumors enhances the specificity of the adenoviral vector compared with the EGR1 promoter. The DF3 promoter can be linked with the E1A gene to drive viral replication specifically in DF3-producing cells and therefore result in a conditionally replication-competent vector. In addition, the DF3 promoter can also be linked downstream to the cDNA for human TNF-α to enhance gene expression of a radio-sensitizing therapeutic cytokine selectively in DF3-producing cells. Replication-competent vectors are advantageous over replication-incompetent vectors in that a reduced number of applications are required to obtain a similar biological effect. Selective TNF-α expression in DF3-producing cancer cells utilizing Ad.DF3.TNF therapy combined with IR may therefore augment local antitumor effects, potentially increasing the number of patients who have a complete pathologic response and subsequently increasing the number of patients who may derive a survival advantage.

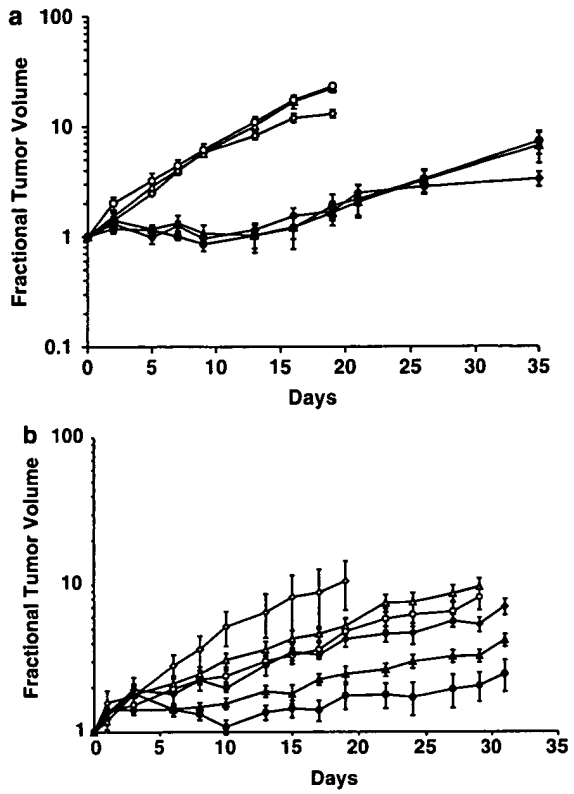

Figure 5 TNF-α expression in Ad.DF3.TNF-treated Bic-1 xenografts leads to an enhanced antitumor response to IR. (a) Athymic nude mice bearing Seg-1 xenografts (mean volume = 245.2 ± 14.2 mm³, n=26) were randomized to one of six treatment groups: intratumoral injection of viral buffer alone (◇), 2x10⁸ pu Ad.DF3.Null (SYMBOL 234 f "MathB" s 11), 2x10⁸ pu Ad.DF3.TNF (○), and three corresponding groups combined with 25 Gy IR (five 5 Gy fractions) (□,□,●). Seg-1 xenografts treated with either Ad.DF3.Null or Ad.DF3.TNF demonstrate no change in regrowth pattern compared with buffer alone. The addition of IR did not alter the regrowth pattern of the vector-treated xenografts compared with buffer-treated tumors. (b) Athymic nude mice bearing Bic-1 xenografts (mean volume = 296.6 ± 20.9 mm³, n=43) were randomized to the same treatment groups. Bic-1 xenografts demonstrate a small reduction in tumor volume following treatment with either Ad.DF3.Null alone or Ad.DF3.TNF alone compared to buffer. The addition of IR to treatment with Ad.DF3.TNF enhanced the antitumor effect of IR producing a delay in tumor regrowth pattern compared to either Ad.DF3.Null + IR or buffer + IR (P < 0.05 after day 22, t-test).

In the present study, we examined the use of a conditionally replication-competent adenovirus containing the DF3 tissue-specific promoter linked with a therapeutic gene encoding TNF-α in two human esophageal adenocarcinoma cell lines: Bic-1, a DF3-producing cell line and Seg-1, a DF3-non-producing cell line. DF3/MUC1 is a high molecular weight glycoprotein that is aberrantly overexpressed in several epithelial tumors including breast, ovarian, pancreatic, colonic, gastric, and esophageal neoplasms. TNF-α has demonstrated antitumor activity against human and rodent cell lines via a direct apoptotic effect *in vitro*[38] and results in central hemorrhagic necrosis and modulation of the host immune system *in vivo*.[39] Animal studies using human tumor xenografts have suggested that TNF-α enhances the tumor's response to radiation.[34] These two elements, the DF3/MUC1 promoter and the TNF-α cDNA, combined in an adenoviral delivery vehicle have produced a vector that is both highly selective and therapeutic in DF3-producing cells and xenografts.

Investigations utilizing a conditionally replication-competent adenoviral vector containing the DF3/MUC1 promoter in a breast carcinoma model have demonstrated selective viral replication in tumor cells expressing the DF3/MUC1 glycoprotein.[20] We have shown that TNF-α production is 15-fold greater in conditioned medium from Bic-1 (DF3/MUC1+) cells and 50-fold greater in tumor homogenates of Bic-1 xenografts compared to Seg-1 (DF3/MUC1−) cells and xenografts. By quantifying TNF-α production, we have indirectly shown that activity of the adenoviral vector containing the DF3/MUC1 promoter, Ad.DF3.TNF, is preferential for the Bic-1 human esophageal cancer cell line compared to the Seg-1 tumor cell line. Additionally, regrowth studies demonstrate that TNF-α production following treatment of Bic-1 xenografts with Ad.DF3.TNF enhances the interactive antitumor response with IR. These findings suggest that the addition of intratumoral Ad.DF3.TNF to conventional antitumor modalities such as radiation may potentiate their therapeutic efficacy in DF3/MUC1-producing tumors.

Transcriptional targeting of tumor cells utilizing a tissue specific-promoter, DF3/MUC1, in adenoviral gene therapy provides a powerful technique to selectively direct viral replication and deliver cDNA segments of TNF-α, a therapeutic gene. A conditionally replication-competent adenoviral vector, Ad.DF3-E1A/CMV-TNF may therefore be used to infect specific tumor cells and thus expose these cells to toxic levels of adenovirus and high levels of therapeutic gene expression following viral replication. Increased TNF-α delivery due to vector infection can potentiate the antitumor effects of conventional DNA-damaging therapies such as IR.

Materials and methods

Cell culture

Bic-1 and Seg-1 human esophageal adenocarcinoma cell lines (gift from Dr David Beer, University of Michigan) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco RL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, L-glutamine, 100 μg/ml streptomycin and 100 units/ml penicillin. The cell line was incubated at 37°C in 7% CO_2.

Indirect immunofluorescence analysis of the DF3/MUC1 antigen

Bic-1 and Seg-1 cells (2 × 10^5) were washed with cold phosphate-buffered saline (PBS), incubated with mAb DF3 (1 μg/ml) at 4°C for 1 h, and then washed with cold PBS. Cells were incubated with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co., St Louis, MO, USA) at 4°C for 1 h, washed with cold PBS, and then analyzed by flow cytometry. Intensity of the fluorescence was determined for 10 000 cells.

TNF-α production in Seg-1 and Bic-1 cells

2 × 10^5 Bic-1 and Seg-1 cells were plated in separate six-well tissue culture dishes overnight. The cells were infected with 0.001 MOI of Ad.DF3-E1/CMV-TNF (Ad.DF3.TNF) for 2 h in serum-free medium. Then the medium was exchanged with 4 ml normal growth

medium. Conditioned medium was then harvested at days 0, 1, 2, and 5 and stored at –80°C. Human tumor necrosis factor alpha enzyme linked immunosorbant assay (hTNF-α ELISA, R&D Systems, Minneapolis, MN, USA) was used to quantify human TNF-α levels within the conditioned medium according to the manufacturer's instructions. Ad.DF3.TNF is a conditionally replication-competent adenoviral vector whereby the E1 gene is under the control of the DF3 promoter. Details of vector structure and production have been previously described.[20]

Generation of xenografts

All animal investigations were performed under approved Animal Care Committee (IACUC) protocols at the University of Chicago. Bic-1 and Seg-1 xenografts were produced by injecting 5×10^6 and 3×10^6 cells, respectively, into the subcutaneous space in the right hind limb of athymic nude mice. Xenografts were measured thrice weekly according to the formula length × width × height/2. Each tumor volume measurement was compared to its volume at day 0 and expressed as a fractional tumor volume.

TNF-α production in Seg-1 and Bic-1 xenografts

Following the generation of Bic-1 ($n = 3$) and Seg-1 ($n = 3$) xenografts, the animals were administered 2×10^8 pu Ad.DF3. TNF directly intratumoral using a Hamilton syringe into five separate sites. The virus was administered as a single dose on day 0. Following CO_2 narcosis, the animals were sacrificed and tumors harvested on day 7. Tumors were snap frozen in liquid nitrogen and stored at –80°C. Tumors were thawed and homogenized in 500 µl protein extraction buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.5, 5 mM EDTA, pH 7.5, 10 µg/ml aprotinin, 5 µg/ml leupeptin, and 100 mM PMSF) utilizing the Brinkman Polytron homogenizer (Kinematica, AG, Littau, Switzerland) for 30 sec on ice. After four freeze/thaw cycles, tumor homogenates were centrifuged at 4°C at 10 000 rpm for 10 min utilizing the RC5C refrigerated centrifuge (Sorvall Instruments Inc., Newtown, CT, USA) with the SS-34 rotor. The pellet was discarded and a standard BioRad macro-protein assay (BioRad Laboratories, Hercules, CA, USA) was performed on the supernatant. Human TNF-α in the supernatant was quantified using hTNF-α ELISA (R&D Systems) according to the manufacturer's instructions.

Xenograft histology

Bic-1 ($n = 6$) and Seg-1 ($n = 6$) xenografts generated in athymic nude mice were randomized to treatment with either Ad. DF3.TNF or Ad.DF3.GFP, the null virus containing green fluorescence protein (GFP) at a dose of 2×10^8 pu and then were followed for 21 days. The mice were sacrificed following CO_2 narcosis and the tumors harvested. Representative tumor sections were dissected and snap frozen in OCT (Tissue Tek; Sakura Finetek USA Inc., Torrance, CA, USA). Sections 4 µm in thickness were cut from the OCT blocks and mounted on poly-L-lysine coated slides (Probe-On, Fisher Scientific, Pittsburgh, PA, USA) and then air dried for 30 min. Sections were hydrated in PBS for 5 min and fixed in 1% methanol-free paraformaldehyde (Polysciences, Warrington, PA, USA). After PBS washes, the tissue section was mounted in a glycerol-based mounting medium and examined for green fluorescence protein or counterstained with DAPI by adding one drop of Vectashield with DAPI (Vector, Burlingame, CA, USA) and then examined for nuclear staining. Sections were stained separately due to the DAPI staining interfering with the GFP expression. Fluorescence was determined with a Zeiss photomicroscope equipped with a Hamamatsu digital camera with QED imaging analysis software for the Apple Macintosh 7600. Photographs were colored in Paint Shop Pro 5.0 (Jasc Software, Minneapolis, MN, USA).

Xenograft regrowth studies

Following the generation of Bic-1 ($n = 46$) and Seg-1 ($n = 46$) xenografts, animals with a mean xenograft volume of 260 mm^3 were randomized to one of six groups: buffer alone ($n = 5$), buffer+radiation ($n = 5$), Ad.DF3. Null ($n = 8$), Ad.DF3.Null+radiation ($n = 10$), Ad.DF3.TNF ($n = 8$), and Ad.DF3.TNF+radiation ($n = 10$). The adenoviral vectors were administered as a single dose of 2×10^8 pu directly within the tumor using a Hamilton syringe in 10 µl buffer through a single entry point to five separate sites. A total of 10 µl buffer alone was administered in a similar manner to animals in the first two groups. Tumor beds were irradiated using a Pantak PMC 1000 X-ray generator (East Haven, CT, USA) operating at 150 kV and 25 mA at a dose rate of 192 cGy/min. Animals were shielded with lead except for the tumor bearing hind limb. The Bic-1 xenografts received a dose of 4 Gy (Gray) each day on days 0, 1, 2, and 3 (total 16 Gy) and the Seg-1 xenografts a dose of 5 Gy each day on days 0, 1, 2, 3, and 4 (total 25 Gy) determined according to their radiosensitivity curves (Seg-1, D_0=86 cGy; Bic-1, D_0=157 cGy). Groups were compared according to their mean fractional tumor volumes.

Statistical analysis

All values are expressed as the mean ± standard error of the mean (s.e.m.). Student's *t*- test was used to determine a statistical significance of 0.05 between mean TNF-α levels in tumor homogenates. Statistical significance between mean tumor volumes of two treatment groups was also demonstrated using Student's *t*-test.

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Selectivity of a replication-competent adenovirus for human breast carcinoma cells expressing the MUC1 antigen

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The *DF3/MUC1* gene is aberrantly overexpressed in human breast and other carcinomas. Previous studies have demonstrated that the *DF3/MUC1* promoter/enhancer confers selective expression of diverse transgenes in MUC1-positive breast cancer cells. In this study, we show that an adenoviral vector (Ad.DF3-E1) in which the *DF3/MUC1* promoter drives expression of E1A selectively replicates in MUC1-positive breast cancer cells. We also show that Ad.DF3-E1 infection of human breast tumor xenografts in nude mice is associated with inhibition of tumor growth. In contrast to a replication-incompetent adenoviral vector that infects along the injection track, Ad.DF3-E1 infection was detectable throughout the tumor xenografts. To generate an Ad.DF3-E1 vector with the capacity for incorporating therapeutic products, we inserted the cytomegalovirus (CMV) promoter upstream of the TNF cDNA. Infection with Ad.DF3-E1/CMV-TNF was associated with selective replication and production of TNF in cells that express MUC1. Moreover, treatment of MUC1-positive, but not MUC1-negative, xenografts with a single injection of Ad.DF3-E1/CMV-TNF was effective in inducing stable tumor regression. These findings demonstrate that the *DF3/MUC1* promoter confers competence for selective replication of Ad.DF3-E1 in MUC1-positive breast tumor cells, and that the antitumor activity of this vector is potentiated by integration of the TNF cDNA.

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Introduction

Recombinant adenoviruses have been used as highly efficient vectors for in vitro and in vivo gene transfer. Adenovirus-mediated gene transduction has been achieved in a broad spectrum of eukaryotic cells, and is independent of cell replication (1, 2). In addition, the *E1* gene-deleted, replication-defective adenoviruses can accommodate large DNA inserts (1, 2). However, limitations of this vector system for cancer therapy have included the nonselective delivery of therapeutic genes to both normal cells and tumor cells. Moreover, the replication-defective adenoviruses are limited by their inability to infect and then spread to neighboring tumor cells. Strategies to circumvent these limitations have involved the use of promoters or enhancers that are specific to or selective for tumor tissue in order to direct replication of the adenovirus in the desired target cells (3). In this context, the minimal promoter/enhancer from the prostate-specific antigen (PSA) gene has been used to drive E1A expression and thereby create an adenovirus, designated CN706, that selectively replicates in PSA-positive cells (4). A similar strategy using the albumin promoter has been used to develop a herpes simplex virus that selectively replicates in hepatoma cells (5).

The DF3/MUC1 antigen is a high-molecular-weight glycoprotein that is aberrantly overexpressed in human breast and other carcinomas (6–8). The *MUC1* gene contains seven exons and has been mapped to chromosome 1q21-24 (9, 10). It spans 4–7 kb's, depending on the number of conserved 60-bp tandem repeats. Overexpression of the *MUC1* gene in human breast cancer cells is regulated at the transcriptional level (11, 12). Cloning and characterization of the 5′ flanking region of the *MUC1* gene has shown that expression of the gene is regulated mainly by sequences between positions -598 bp and -485 bp upstream from the transcription start site (13). The *MUC1* promoter/enhancer region has been shown in the context of retroviral vectors to direct expression of prodrug-activating enzymes and to confer selective killing of MUC1-positive human carcinoma cells (14). In other studies with replication-defective adenoviruses, the *MUC1* promoter has been used to selectively express β-galactosidase (β-gal) or herpes simplex virus thymidine kinase (HSV-tk) in MUC1-positive breast cancer cells (15).

In this study, we have constructed an adenovirus in which E1A sequences are expressed under control of the *MUC1* promoter. The results demonstrate that the virus replicates selectively in MUC1-positive cells in vitro. We also demonstrate that the virus is effective in expressing the *TNF* transgene and in treating MUC1-positive breast cancer xenografts in nude mice.

Methods

Cells and cell culture. The following cells were obtained from the American Type Culture Collection (Manassas, Vir-

ginia, USA): MCF-7, ZR-75-1, BT-20, and MDA-MB-231 human breast cancer cell lines; the Hs578Bst myoepithelial cell line derived from normal breast tissue adjacent to an infiltrating ductal carcinoma; the PA-1 human ovarian cancer cell line; and the human embryonic kidney (HEK) 293 cell line. MCF-7, MDA-MB-231, Hs578Bst, and HEK 293 cells were cultured in DMEM. ZR-75-1 cells were grown in RPMI 1640 medium supplemented with 25 mM HEPES. The BT-20 and PA-1 cell lines were grown in Eagle's MEM. All media were supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin.

Structure of the DF3 promoter replication-competent adenovirus. A 480-bp portion of the *E1* gene was generated from pKSCMVE1 (16) using the primers A2s(475)XS (5′-GGACTAGTAAGCTTCTCCAGCCCGTGAGTTCCTCAAGAGG-3′) and A2a(921)NS (5′-TCCCCCGGGCTAGCATCGATCACCTCCGGCACAA-3′). The 480-bp fragment was digested with *SpeI* and *SmaI* restriction enzymes and then ligated into pAdClxB (plasmid 1) (16). The DF3 promoter region was amplified from pDF3 using primer DF3.5′ (5′-TCTAGACTAGTGTGGACCCTAGGGTTCATCGGAG-3′) and DF3.3′ (5′-AACTCGAGGATTCAGGCAGGCGCTGGCT-3′). The resulting 780-bp fragment was ligated into plasmid 1 using *SpeI* and *XhoI* restriction sites to generate plasmid 2. The remaining E1A sequence (~5 kb) was digested from the pKSCMVE1 region and cloned into plasmid 2 using complementary *ClaI* restriction enzyme sites (pDF3-E1/CMV). The cytomegalovirus (CMV) promoter sequence was then deleted to generate pDF3-E1, which contains the DF3 promoter to drive the *E1A* gene. A marker plasmid was constructed by inserting green fluorescent protein (GFP) cDNA into pDF3-E1/CMV at the *Bam*HI site (pDF3-E1/CMV-GFP). Construction of pDF3-E1/CMV-TNF was performed by substituting GFP with the TNF cDNA sequence (17).

Replication-competent adenovirus under control of the DF3 promoter was prepared by standard homologous recombination techniques using the adenoviral packaging plasmid pJM17 (kindly provided by F. Graham, McMaster University, Hamilton, Ontario, Canada) in HEK 293 cells. Three micrograms of each shuttle plasmid was mixed with 6 μg pJM17 and precipitated with $CaCl_2$. This was used to transfect HEK 293 cells. Recombinant adenovirus was isolated from a single plaque and expanded in HEK 293 cells. DNA was purified and analyzed by PCR.

Virus was prepared by infecting eighty 15-cm plates of HEK 293 cells and harvesting the detached cells after 48 hours. The virus remained associated with the cells. Cells were collected by centrifugation at 400 *g* for 5 minutes at 4°C. The cells were resuspended in 10 mL of cold PBS (free of Ca^{2+} and Mg^{2+}), and were lysed with three cycles of freeze and thaw. Cells were collected by centrifugation at 1,500 *g* for 10 minutes at 4°C. The supernatant was placed on a gradient prepared with equal parts of CsCl in PBS (1.45 g/mL and 1.20 g/mL), and then centrifuged for 2 hours at 15,000 *g* at 12°C. The virus band was removed, rebanded in a preformed CsCl gradient by ultracentrifugation for 18 hours, and dialyzed into cold 10 mM Tris-HCl (pH 7.4) containing 10 mM $MgCl_2$ and 10% glycerol. Titers of purified adenovirus were determined by spectrophotometry and by plaque assays.

The shuttle plasmids pCMV-LacZ and pCMV-TNF were used to generate the Ad.CMV-β-gal and Ad.CMV-TNF recombinant adenoviruses in which expression of LacZ or TNF is driven by the CMV promoter. Ad.DF3-β-gal was prepared as described (15).

Indirect immunofluorescence analysis of MUC1 antigen. Cells (2×10^5) were washed extensively with cold PBS, incubated with mAb DF3 (1 μg/mL) at 4°C for 1 hour, and washed with cold PBS. Cells were incubated with FITC-conjugated goat anti-mouse IgG (Sigma Chemical Co., St. Louis, Missouri, USA) at 4°C for 1 hour, washed with cold PBS, and then analyzed by flow cytometry. Intensity of fluorescence was determined for 10,000 cells.

Western blot analysis of Ad2 E1A and Ad5 E1B expression. Three days after viral infection, cells were lysed in cell lysis buffer (50 mM HEPES at pH 7.5, 0.15 M NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 10 mM NaF, 10 ng/mL aprotinin, 10 ng/mL leupeptin, 1 mM DTT, and 1 mM sodium vanadate), incubated for 30 minutes on ice, and centrifuged at 1,500 *g* for 10 minutes at 4°C. The supernatants were transferred to Eppendorf tubes and heated at 100°C for 5 minutes. Protein was analyzed by immunoblotting with mAb Ad2 E1A or Ad5 E1B (1 μg/mL; Oncogene Research Products, Cambridge, Massachusetts, USA). Reactivity was visualized by enhanced chemiluminescence (Amersham Life Sciences Inc., Arlington Heights, Illinois, USA).

In vitro viral replication assay. Monolayer cell cultures in 24-well dishes (5×10^4 cells/well) were infected with Ad.DF3-E1, Ad.DF3-E1/CMV, Ad.DF3-E1/CMV-TNF, or wild-type Ad5 at an moi of 1.0 plaque-forming units (pfu) per well. Virus inocula were removed after 2 hours. The cells were then washed twice with PBS and incubated at 37°C for varying periods of time. Lysates were prepared with three cycles of freeze and thaw. Serial dilutions of the lysates were titered on HEK 293 cells.

Cytopathic effect assays. Cells were prepared 24 hours before infection with adenoviruses at the indicated

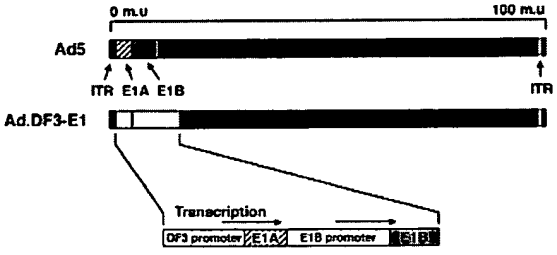

Figure 1
Structure of Ad.DF3-E1. ITR, inverted terminal repeat; m.u., map unit.

moi. Photomicrographs were taken on days 3, 5, and 7 after infection.

ELISA. On days 1, 3, 5, and 7 after viral infection, supernatants were assayed for TNF levels using the Human Tumor Necrosis Factor ELISA kit from Sigma Chemical Co.

In vivo gene transfer to human breast cancer xenografts. Female athymic nude mice (Taconic Farms, Germantown, New York, USA) aged 5–6 weeks were implanted subcutaneously with a pellet of estradiol-17β (1.7 mg, 60-day release; Innovative Research of America, Sarasota, Florida, USA) 1 day before tumor inoculation. Tumors were established by subcutaneous injection of 10^7 MCF-7 cells or MDA-MB-231 cells in 0.2 mL of 50% Matrigel (Becton Dickinson and Co., Franklin Lakes, New Jersey, USA) and 50% PBS into the flanks of mice. Tumors were allowed to grow to approximately 6–7 mm in diameter. For intratumor injection, 30 μL of virus suspension in PBS was injected using a 25-gauge needle. Tumors were measured at the indicated times after injection in their longest dimension and at 90 degrees to that measurement. Tumor volumes were calculated using this formula: (length × width^2)/2. Tumor volumes were normalized to 100% on day 0 (V/V_0). Results are expressed as the fractional tumor volume (mean ± SD). Statistical significance was assessed with the Student's *t* test.

Histopathological analysis. At 4–5 weeks after tumor implantation, up to 2×10^8 pfu of purified adenovirus were injected into MCF-7 tumor xenografts using a Hamilton syringe with a 26-gauge needle. The needle was coated with fine charcoal particles to mark the needle track. Tumors were sectioned for analysis of reporter gene expression.

Results

Selective replication of an adenovirus in MUC1-positive cells in vitro. To construct an adenovirus that replicates selectively in MUC1-positive cells, we placed the *DF3/MUC1* promoter upstream of the E1 region. Ad.DF3-E1 was constructed by inserting the expression cassette at the deleted E1 region of the replication-defective Ad5 virus (Figure 1).

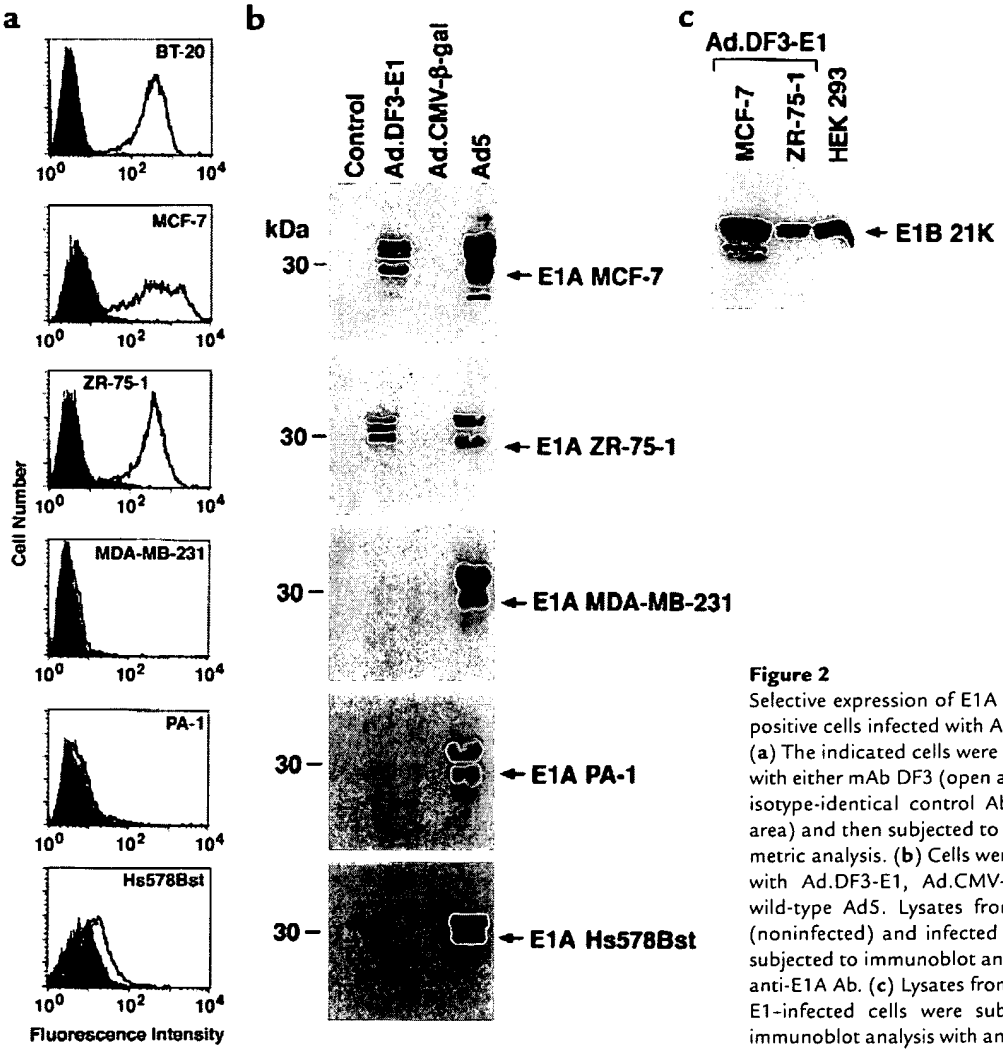

Figure 2
Selective expression of E1A in MUC1-positive cells infected with Ad.DF3-E1. (**a**) The indicated cells were incubated with either mAb DF3 (open area) or an isotype-identical control Ab (shaded area) and then subjected to flow cytometric analysis. (**b**) Cells were infected with Ad.DF3-E1, Ad.CMV-β-gal, or wild-type Ad5. Lysates from control (noninfected) and infected cells were subjected to immunoblot analysis with anti-E1A Ab. (**c**) Lysates from Ad.DF3-E1–infected cells were subjected to immunoblot analysis with anti-E1B.

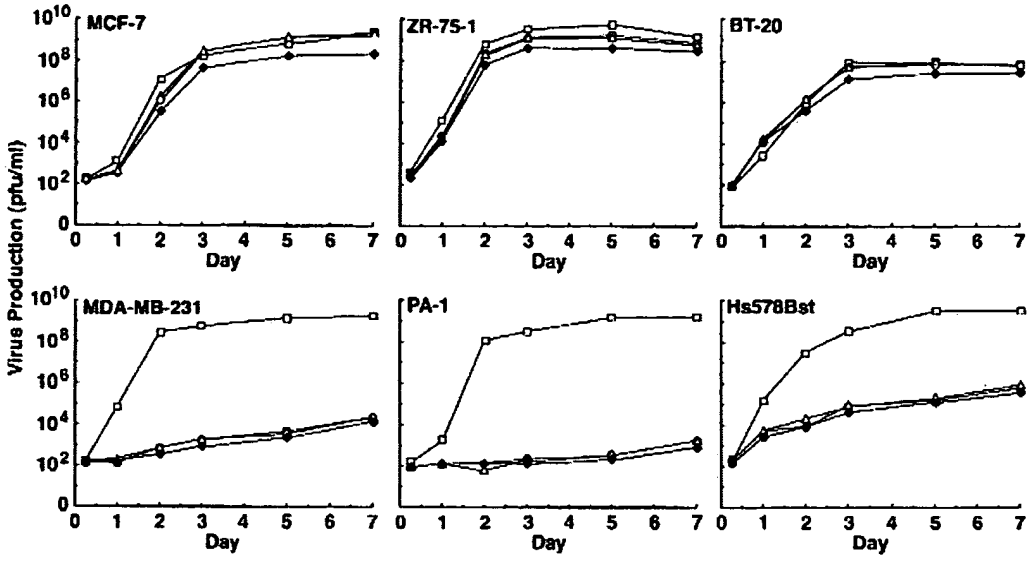

Figure 3
Replication efficiency of Ad.DF3-E1 (open circles), Ad.DF3-E1/CMV (open triangles), Ad.DF3-E1/CMV-TNF (filled diamonds), and wild-type Ad5 (open squares) in human cell lines. Monolayers in 24-well plates were infected at an moi of 1.0 pfu per cell. Virus production was assessed by plaque assays.

Human cell lines were used to assess the selectivity of Ad.DF3-E1 viral replication. As determined by flow cytometry with an anti-MUC1 Ab, the MCF-7, ZR-75-1, and BT-20 breast cancer cells expressed MUC1 (Figure 2a). The MDA-MB-231 breast cancer cells and PA-1 ovarian cancer cells, by contrast, exhibited little if any MUC1 expression and were used as controls (Figure 2a). Moreover, MUC1 expression was detectable, but decreased, in breast Hs578Bst epithelial cells compared with that in the breast cancer cell lines (Figure 2a). The cells were infected with Ad.DF3-E1 at an moi of 10 for a period of 2 hours, and then washed and resuspended in medium. E1A protein expression was assessed by immunoblot analysis. The results demonstrate expression of E1A in the MUC1-positive MCF-7 and ZR-75-1 cells, but not in the MUC1-negative MDA-MB-231 and PA-1 cells (Figure 2b). There was also little if any detectable E1A expression in Ad.DF3-E1–infected Hs578Bst cells (Figure 2b). As controls, the cells were also infected with a replication-defective Ad5 virus expressing β-gal under control of the CMV promoter (Ad.CMV-β-gal) or with wild-type Ad5. Whereas there was no detectable E1A expression in cells infected with Ad.CMV-β-gal, E1A was expressed by each of the cells infected with wild-type Ad5 (Figure 2b). Expression of the *E1B* gene was also detectable in MUC1-positive, but not MUC1-negative, cells infected with Ad.DF3-E1 (Figure 2c and data not shown). These findings indicate that infection with Ad.DF3-E1 results in selective expression of E1A in breast cancer cells.

Viral titers were assessed by examining plaque formation to evaluate Ad.DF3-E1 replication in the different cell lines. In MUC1-positive MCF-7 cells, ZR-75-1 cells, and BT-20 cells, Ad.DF3-E1 replicated at levels comparable to that of wild-type Ad5 (Figure 3). By contrast, compared with Ad5, the titer of Ad.DF3-E1 was reduced by 5–6 logs in MUC1-negative MDA-MB-231 and PA-1 cells, and by 4–5 logs in Hs578Bst cells (Figure 3). To determine whether Ad.DF3-E1 induces selective cell lysis, we infected MCF-7 cells, PA-1 cells, and Hs578Bst cells at moi's of 0.01, 0.1, and 1.0. Plaque formation was assessed on days 3, 5, and 7. Although the MCF-7 cells displayed Ad.DF3-E1–induced lysis (Figure 4a), there were no apparent cytopathic effects of Ad.DF3-E1 on PA-1 (Figure 4b) or Hs578Bst (Figure 4c) cells. These results are in concert with the cell-dependent expression of E1A, and demonstrate selective replication of Ad.DF3-E1 in MUC1-positive breast cancer cells.

Treatment of breast tumor xenografts with Ad.DF3-E1. To evaluate the therapeutic efficacy of Ad.DF3-E1 in vivo, MCF-7 and MDA-MB-231 breast tumor xenografts were established in nude mice and then injected once with 2×10^8 pfu of Ad.DF3-E1. As controls, tumors were injected with either PBS or the replication-defective Ad.DF3-β-gal virus. Infection with Ad.DF3-E1 was associated with inhibition of MCF-7 tumor growth (Figure 5a). By 4 weeks after Ad.DF3-E1 injection, the tumors had regressed to being barely palpable (Figure 5a). These findings were in contrast to the progressive growth of tumors injected with PBS or Ad.DF3-β-gal (Figure 5a). By contrast, Ad.DF3-E1 had no apparent effect on growth of MDA-MB-231 tumors (Figure 5b). These results indicate that a single injection of Ad.DF3-E1 results in selective cytolysis and regression of an MUC1-positive tumor.

Distribution of Ad.DF3-E1 in tumors. An obstacle to using gene therapy to treat cancer is the difficulty in distributing the vector throughout the tumor mass. The finding that Ad.DF3-E1 induces tumor regression supports the spread of this virus beyond the initial infection site. To assess viral distribution and to generate competent Ad.DF3-E1 viruses that have the capacity to incorporate additional transgenes, the CMV promoter was inserted upstream of the gene expressing GFP (Figure 6a). Infection of MCF-7 cells with Ad.DF3-E1/CMV-GFP (moi = 10) was associated with little GFP expression at 36 hours (Figure 6b). However, by 72 hours after infection (a period sufficient for viral replication), there was clearly detectable GFP expression and induction of cytopathic effects (Figure 6b and data not shown). A similar infection of PA-1 cells resulted in significantly less GFP expression at 72 hours, with no evidence of lysis (Figure 6b and data not shown). The Ad.DF3-E1/CMV-GFP virus was also injected into MCF-7 tumor xenografts to assess viral distribution. As a control, the tumor was injected with the replication-defective Ad.DF3-β-gal virus. Staining of the tumor for β-gal expression demonstrated Ad.DF3-β-gal infection only along the needle track (Figure 6c). By contrast, GFP expression was detectable throughout the tumor mass (Figure 6c). These findings demonstrate that the competent Ad.DF3-E1 virus, but not the replication-defective virus, spreads throughout the tumor.

Expression of TNF by Ad.DF3-E1. Construction of the MUC1-competent Ad.DF3-E1 virus with the capacity to express additional transgenes allows for the insertion of various genes encoding therapeutic products. TNF was selected as an initial candidate because of its direct antitumor activity (18). Ad.DF3-E1/CMV-TNF virus was constructed by substituting GFP with TNF sequences (Figure 7a). To assess the effects of the CMV promoter on transcription driven by the DF3 promoter, we compared E1A expression in MUC1-positive MCF-7 and ZR-75-1 cells that were infected with either Ad.DF3-E1 or Ad.DF3-E1/CMV-TNF. The results demonstrate that the CMV promoter has little if any effect on transcription driven by the DF3 promoter (Figure 7b). Ad.DF3-E1/CMV-TNF infection of MCF-7 cells, but not PA-1 cells, was also associated with cytopathic effects (data not shown). In addition, growing the Ad.DF3-E1/CMV-TNF virus in MUC1-positive cells resulted in titers that were 4–5 logs higher than those of the virus grown in MUC1-negative cells. These higher titers supported replication that is, like that of Ad.DF3-E1, dependent on MUC1 expression (data not shown). Consistent with these findings, infection of MCF-7 cells with Ad.DF3-E1/CMV-TNF was associated with production of TNF that was 10^6-fold higher

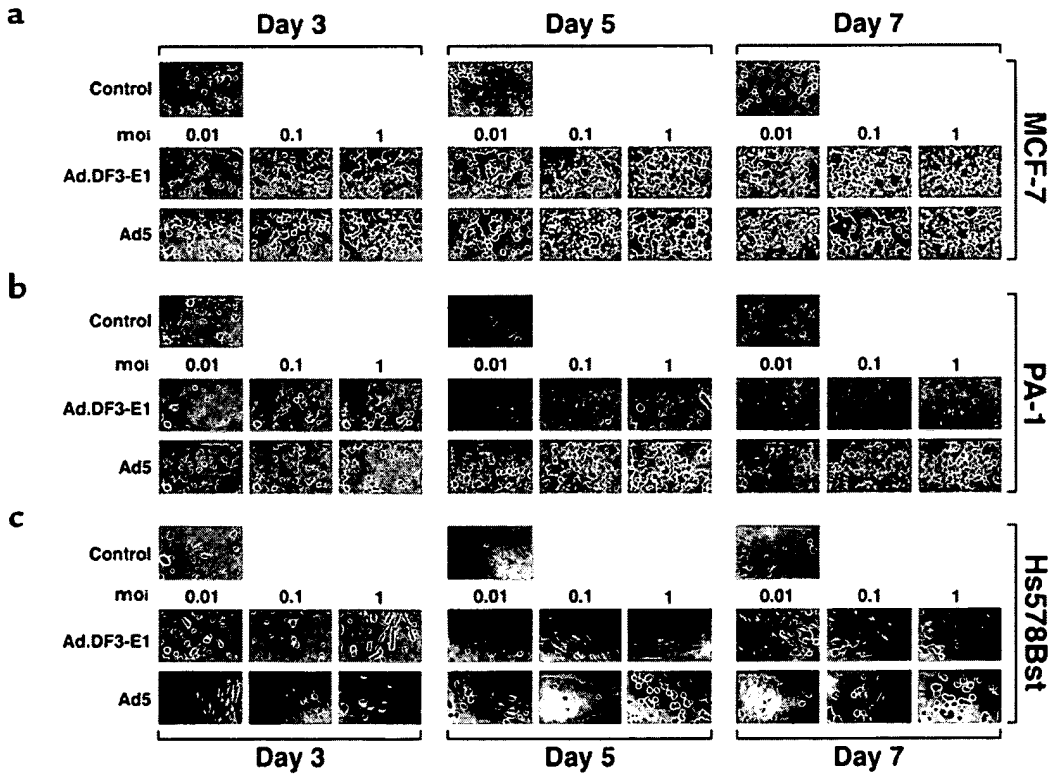

Figure 4
Cytopathic effects associated with Ad.DF3-E1 infection. MCF-7, PA-1, and Hs578Bst cells were infected with either Ad.DF3-E1 or wild-type Ad5 at the indicated moi. Photomicrographs were obtained at the indicated times after infection. ×200.

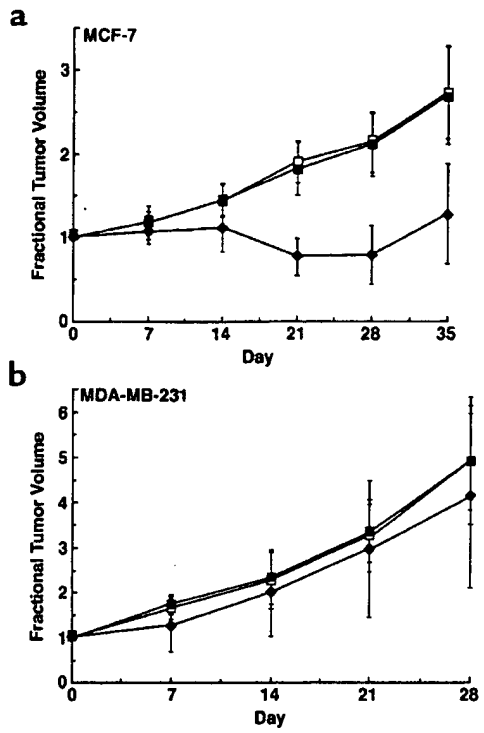

Figure 5
Effects of Ad.DF3-E1 on growth of MCF-7 and MDA-MB-231 tumor xenografts in nude mice. MCF-7 (a) or MDA-MB-231 (b) tumor xenografts were grown subcutaneously to volumes of 150–200 mm^3. Groups of mice (n = 5) were treated with 2×10^8 pfu of Ad.DF3-E1 (filled diamonds) or Ad.DF3-β-gal (filled squares) by intratumoral injection on day 0. An equal volume of PBS was injected as a control (open squares). Tumors were measured weekly. The results are expressed as fractional tumor volume (V/V_0). MCF-7 tumors infected with Ad.DF3-E1 were significantly smaller than those treated with PBS or Ad.DF3-β-gal at day 21 ($P < 0.001$), day 28 ($P < 0.001$), and day 35 ($P < 0.01$).

than that obtained after infection with Ad.CMV-TNF (Table 1). In addition, TNF production by Ad.DF3-E1/CMV-TNF–infected MCF-7 cells was also 10^5- to 10^6-fold higher than that obtained by a similar infection of PA-1 cells (Table 1). These results demonstrate that Ad.DF3-E1/CMV-TNF is selective for MUC1-positive cells and that it expresses the *TNF* transgene.

Treatment of human breast tumor xenografts with Ad.DF3-E1/CMV-TNF. MUC1-positive MCF-7 and MUC1-negative MDA-MB-231 tumors were established in nude mice to assess treatment with Ad.DF3-E1/CMV-TNF. Mice bearing tumors of 150–200 mm^3 were injected intratumorally with 10^8 pfu of virus in PBS. Compared with injections of PBS alone, treatment of MDA-MB-231 tumors with Ad.DF3-E1/CMV-TNF resulted in growth-inhibiting effects (Figure 8a). Similarly, treatment of MCF-7 cells with the defective Ad.CMV-TNF virus was associated with partial suppression of growth (Figure 8b). These results indicate that expression of TNF in the context of a replication-incompetent virus is insufficient to induce tumor regression. In contrast to these findings, treatment of MCF-7 tumors with Ad.DF3-E1/CMV-TNF, but not Ad.DF3-E1/CMV-GFP, was associated with regression to barely palpable tumors (Figure 8b). Four of the five animals were followed for longer periods; one animal exhibited tumor regrowth, and the other three had barely palpable tumors (Figure 8c). Retreatment of the recurrent tumor with Ad.DF3-E1/CMV-TNF on day 105 was associated with subsequent regression (Figure 8c). These findings indicate that antitumor activity is potentiated in the setting of a virus that exhibits selective competence for replication and expression of the *TNF* transgene.

Discussion

Nearly 80% of primary human breast carcinomas express high levels of MUC1 antigen (6). Other studies have shown that human breast tumors express the *MUC1* gene at the mRNA and protein levels in approximately 30-fold greater amounts than are found in normal breast tissue and benign lesions (19). These findings and the demonstration that MUC1 expression is regulated at the transcriptional level in breast tumor cells in culture (11) have supported activation of the *MUC1* gene in transformed mammary epithelium. Although studies of MUC1 expression have been largely focused on breast tumor cells, other work has demonstrated that MUC1 is overexpressed in diverse carcinomas, including ovarian (8), prostate (20), pancreas (21), and lung cancers (22). On the basis of these findings, and considering the potential for identifying elements in the *MUC1* promoter that are activated in carcinomas, sequences responsible for *MUC1* transcription were cloned from the 5′ flanking region of the *MUC1* gene (13). Subsequent work using retroviral and adenoviral vectors demonstrated that the *MUC1* promoter is functional in directing selective and efficient expression of heterologous genes in MUC1-positive cells (14, 15). In vivo experiments using breast tumor implants in nude mice injected with Ad.DF3-β-gal demonstrated that β-gal expression is limited to the MUC1-positive xenografts, predominantly along

Table 1
TNF production in MCF-7 and PA-1 cells

Virus	Ad.DF3-E1		Ad.DF3-E1/CMV-TNF		AD.CMV-TNF	
moi	0.1	1.0	0.1	1.0	0.1	1.0
MCF-7						
Day 1	0	0	77	128	0	51
3	0	0	3,218	22,454	77	103
5	0	0	530,965	101,352,000	128	154
7	0	0	101,352,000	101,352,000	77	103
PA-1						
Day						
1	0	0	0	25	103	128
3	0	0	77	103	25	51
5	0	0	103	412	77	180
7	0	0	77	1,467	25	128

Results are expressed as TNF concentration (pg/mL).

the needle track (15). Moreover, in a model of intraperitoneal breast cancer metastases, treatment with Ad.DF3-HSV-tk and ganciclovir (GCV) resulted in inhibition of tumor growth (15).

A significant obstacle to cancer gene therapy, even after direct intratumoral administration, is the limited distribution of the vector within the tumor mass. Coadministration of replication-defective virus expressing suicide genes with wild-type virus (23, 24) or with genes essential for replication (25) has been explored to increase transduction efficiency. Direct injection of retroviral producer cells into brain tumors has also been performed to achieve intratumoral vector production (26). Other studies with an *E1B* gene-deleted adenovirus have demonstrated selective replication in p53-mutant tumor cells and antitumor activity (27–30). Conflicting results, however, have been reported in regard to the relationship between replication of the *E1B*-deleted virus and p53 status (31–33). Our studies have used yet another strategy to increase transduction efficiency. The results demonstrate that insertion of the MUC1 promoter upstream of the *E1A* gene confers selective expression of these proteins in MUC1-positive cells. The results also demonstrate that the Ad.DF3-E1 virus induces selective lysis of tumor cells that express MUC1. Of importance, the replication-competent Ad.DF3-E1 virus spread throughout the tumor mass, whereas the replication-defective Ad.DF3-β-gal was restricted to infection of cells along the needle track. These findings indicate that Ad.DF3-E1 and other vectors with the capacity to selectively replicate in tumors have the potential for greater efficacy than that achieved with replication-defective viruses.

Insertion of the PSA promoter into Ad5 to drive expression of E1A has generated a virus known as CN706, which is competent for replication in PSA-positive prostate cancer cells (4). CN706 has been further engineered to include the human glandular kallikrein (hK2) promoter to induce expression of the *E1B* gene (34). The use of both promoters in the CV764 virus has resulted in further attenuation of growth in nonprostate cancer cells (34). In our current study with Ad.DF3-E1, the MUC1 promoter has been used to drive expression of *E1A*. Replication of Ad.DF3-E1 in MUC1-positive cells was increased by 5–6 logs over that obtained in MUC1-negative cells. The selectivity of Ad.DF3-E1 replication in MUC1-positive cells is comparable to that of CV764 in prostate cells (34). In vivo studies with the CN706 virus have further demonstrated lysis of PSA-positive tumor xenografts (4). Similar findings of tumor regression were obtained in our current studies with Ad.DF3-E1 and MUC1-positive MCF-7 xenografts. By contrast, injection of MCF-7 tumors with a replication-defective virus had little if any effect on tumor growth. Moreover, injection of MUC1-negative MDA-MB-231 tumors with Ad.DF3-E1 had little effect on tumor growth. These findings support selective repli-

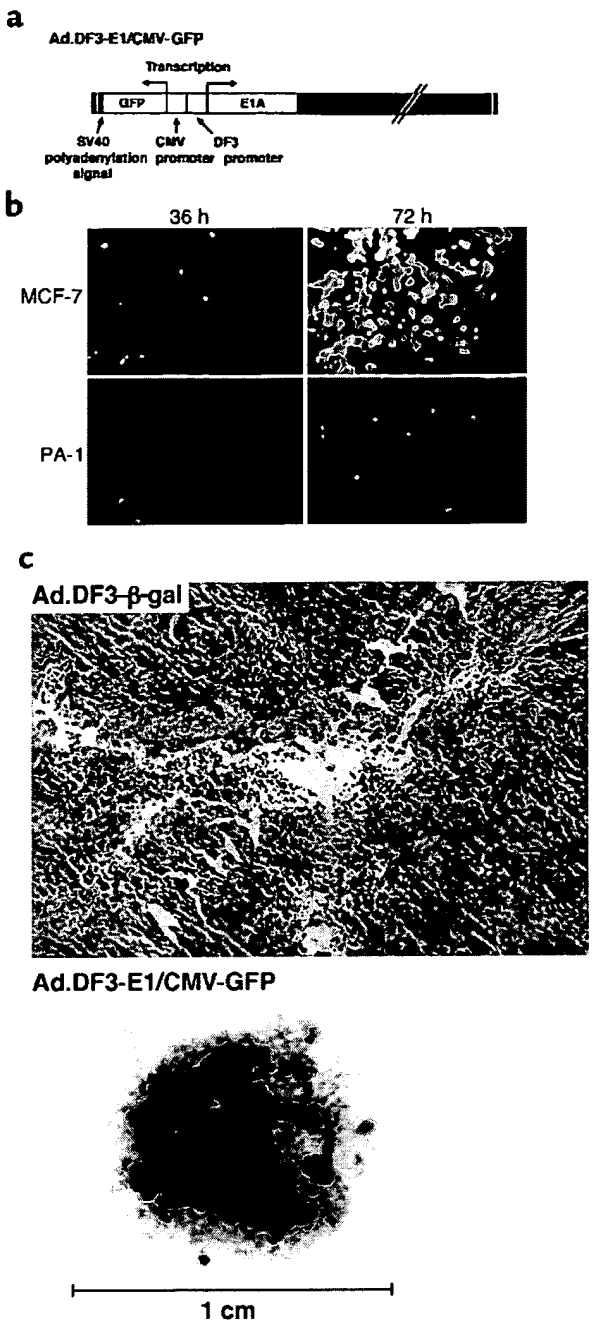

Figure 6
Characterization and intratumoral distribution of Ad.DF3-E1/CMV-GFP. (**a**) Structure of Ad.DF3-E1/CMV-GFP. (**b**) MCF-7 cells and PA-1 cells were infected with Ad.DF3-E1/CMV-GFP at an moi of 10. GFP expression was assessed by photomicrographic examination at 36 hours and 72 hours after infection. (**c**) MCF-7 tumor xenografts (150 mm^3) were injected with 2×10^8 pfu of Ad.DF3-β-gal (upper panel) or Ad.DF3-E1/CMV-GFP (lower panel). At 21 days after injection, the tumors were removed, embedded in OCT (Tissue-Tek; Sakura Finetek USA Inc., Torrance, California, USA), and frozen on dry ice. They were then cryosectioned with a microtome. Sections were fixed in 0.5% glutaraldehyde and stained with X-gal (upper panel; ×200). Sections shown in lower panel were visualized by STORM (Molecular Dynamics, Sunnyvale, California, USA).

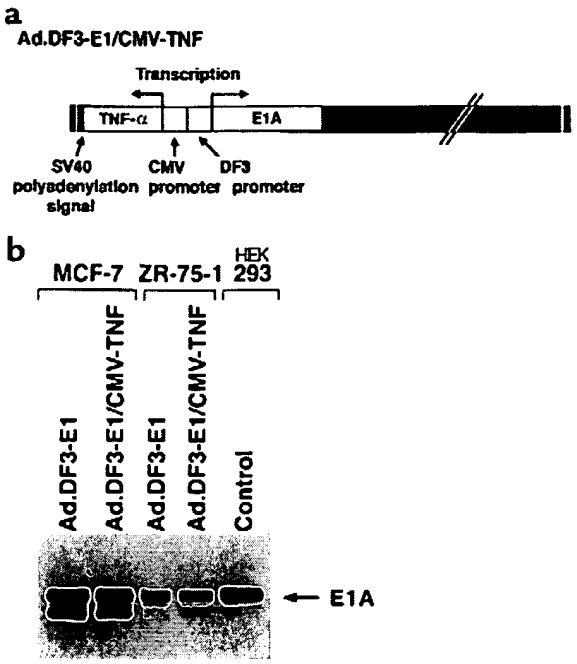

Figure 7
Characterization of Ad.DF3-E1/CMV-TNF. (**a**) Structure of Ad.DF3-E1/CMV-TNF. (**b**) Cells were infected with Ad.DF3-E1 or Ad.DF3-E1/CMV-TNF. Lysates were subjected to immunoblot analysis with anti-E1A.

cation of Ad.DF3-E1 in MUC1-positive tumors in vivo and, thereby, lysis of the tumor cells.

E1B-deleted adenoviruses, which are replication competent in cancer cells (27, 28), have been engineered to express the *HSV-tk* gene and to thereby sensitize tumors to GCV (35, 36). Treatment of tumor xenografts with the replication-competent Ad.TK-RC virus and GCV has been shown to prolong survival over that obtained with Ad.TK-RC alone (36). These results indicate that the lysis caused by the replicating virus and the suicide/prodrug therapy with HSV-tk/GCV results in improved antitumor activity. In the construction of Ad.DF3-E1, we inserted the CMV promoter in a direction opposite to that of the MUC1 promoter to drive additional transgenes. Importantly, the CMV promoter had no detectable effect on activity of the DF3 promoter. Placement of the *GFP* gene downstream of the CMV promoter resulted in the generation of Ad.DF3-E1/CMV-GFP, which replicates in MUC1-positive cells and expresses GFP. Although GFP could be replaced in this vector by *HSV-tk* or other suicide genes, we selected the *TNF* gene because of the selective antitumor activity of the TNF protein (18). Infection of MUC1-positive tumor cells with the Ad.DF3-E1/CMV-TNF virus was associated with viral replication and selective expression of TNF as a consequence of viral production. In this context, both viral replication and TNF production were approximately 5 logs higher in MUC1-positive cells than in MUC1-negative cells. The results also demonstrate that treatment of MUC1-positive MCF-7 cells with Ad.DF3-E1/CMV-TNF resulted in prolonged tumor regression compared with that obtained with Ad.DF3-E1 or Ad.CMV-TNF. In addition, Ad.DF3-E1/CMV-TNF had little if any effect on growth of MUC1-negative MDA-MB-231 tumors. These findings indicate that the Ad.DF3-E1/CMV-TNF virus is competent for replication in MUC1-positive tumors and that it confers improved antitumor activity by expressing the TNF protein.

In summary, replication-competent viruses offer certain advantages over replication-defective vectors for cancer gene therapy when replication is controlled by tumor-selective regulatory sequences. The replication-competent viruses have the capacity to spread throughout the tumor mass and to express therapeutic gene

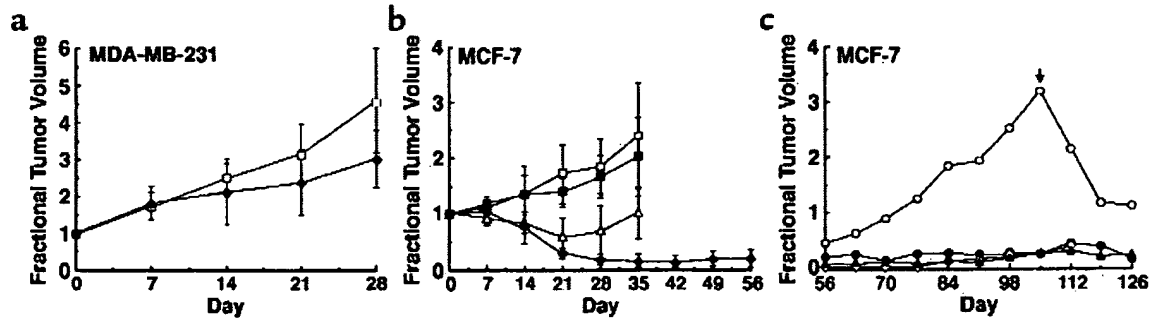

Figure 8
Antitumor effects of Ad.DF3-E1/CMV-TNF. MDA-MB-231 and MCF-7 tumor xenografts were grown subcutaneously in nude mice to volumes of 150–200 mm^3. (**a**) Groups of mice ($n = 5$) bearing MDA-MB-231 tumors were injected intratumorally with PBS (open squares) or 10^8 pfu of Ad.DF3-E1/CMV-TNF (filled diamonds) on day 0. (**b**) Groups of mice ($n = 5$) bearing MCF-7 tumors were injected intratumorally with PBS (open squares), 10^8 pfu of Ad.CMV-TNF (filled squares), 10^8 pfu of Ad.DF3-E1/CMV-TNF (filled diamonds), or 10^8 pfu of Ad.DF3-E1/CMV-GFP (open triangles) on day 0. (**c**) Mice bearing MCF-7 tumors injected with Ad.DF3-E1/CMV-TNF were followed for more than 56 days. One mouse died on day 52 without any tumors. Of the remaining four mice, one exhibited tumor regrowth (open circles). This mouse was reinjected with 10^8 pfu of Ad.DF3-E1/CMV-TNF on day 105 (arrow). The results are expressed as fractional tumor volume (V/V_0). Differences among the MDA-MB-231 treatment groups were not significant. MCF-7 tumors infected with Ad.DF3-E1/CMV-TNF were significantly smaller at day 35 than were those treated with Ad.CMV-TNF ($P < 0.001$) or Ad.DF3-E1/CMV-GFP ($P < 0.01$).

products. Our results demonstrate that the MUC1 promoter confers competence for selective replication of Ad.DF3-E1 in MUC1-positive tumor cells, and that this vector can be used to express additional transgenes. Because MUC1 is overexpressed in diverse human carcinomas, recombinant Ad.DF3-E1 vectors are being developed that express other candidate therapeutic genes, including those encoding *HSV-tk* and cytosine deaminase.

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